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Production of engineered IgM-binding single-chain antibodies in *Escherichia coli*

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SUMMARY

Two single-chain antibodies were engineered and tested as novel binding proteins with specificity for immunoglobulin M. Genes for the two single-chain Fv proteins were assembled from the variable light chain cDNA and variable heavy chain cDNA of monoclonal antibodies DA4.4 and Bet 2, which specifically bind human IgM and mouse IgM, respectively. Both single-chain Fv proteins were designed with a 14-amino acid linker which bridged the variable light chain and variable heavy chain domains. The two proteins were expressed in *Escherichia coli*, purified and assayed for IgM-binding activity. Both proteins demonstrate a binding specificity for their corresponding IgM which is similar to the monoclonal antibodies from which they were derived. These small IgM-binding proteins may have applications in the investigation of the immune response and in the detection and purification of monoclonal antibodies, cell-associated antibodies, and IgM from serum.

INTRODUCTION

Immunoglobulin M (IgM) is produced as a first response to the presentation of antigens in mammals and accounts for 5–10% of the total immunoglobulins [7]. Many mouse monoclonal antibodies (Mabs) and autoantibodies are of the IgM class. Bacterial immunoglobulin G-binding proteins, such as staphylococcal protein A and streptococcal protein G, do not bind or bind poorly to IgM and attempts to identify bacterial IgM receptors by large-scale screening of microbial collections have not yet proven successful [11]. The replacement of anti-IgM Mab reagents with smaller affinity proteins which specifically and reversibly bind the mu chain of IgM would expedite basic research on IgM structure and function and would also benefit the development of IgM-specific separations, diagnostics and therapeutics [2,4,7,13,15].

The variable region (Fv) of a monoclonal antibody maintains the binding specificity and affinity of the whole antibody. Single-chain Fv (sFv) proteins are recombinant polypeptides, composed of an antibody variable light chain (V_L) joined to a variable heavy chain (V_H) by a designed peptide which links the carboxyl (or amino) terminus of the V_L to the amino (or carboxyl) terminus of the V_H [3,8]. Over 15 diverse sFv proteins produced in *E. coli* have been reported to retain the same specificity and similar affinity for their antigens as their parent monoclonal antibodies [16].

We describe in this report the construction and characterization of two sFv proteins with IgM specificity. We have isolated the genes of the variable regions from an anti-human IgM Mab (DA4.4, ATCC HB57) and an anti-mouse IgM Mab (Bet 2, ATCC HB88). Engineered sFv versions of these antibodies expressed in *E. coli* were shown by competitive ELISA to be IgM-specific binding proteins.

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MATERIALS AND METHODS

Isolation of cDNA clones for DA4.4 and Bet 2 V_L and V_H chains

Total RNA was extracted from DA4.4 and Bet 2 hybridoma cells and purified polyadenylated [poly (A+)] RNA was used in cDNA synthesis as described by Gubler and Hoffman [6].

Synthesis of the first strand of cDNA was primed using oligonucleotides with sequences selected from the immunoglobulin data base [9] of constant region domains adjacent to the variable domains as follows: 5′ TGGATGGTGGGAAGATG 3′ (DA4.4 V_L); 5′ ATGGAGTTAGTTTGGGCA 3′ (DA4.4 V_H); 5′ TGGATGGTGGGAAGAT 3′ (Bet 2 V_L); and 5′ GGCCAGTGGATAGAC 3′ (Bet 2 V_H). Libraries were generated by ligation of blunt end cDNA fragments into the *Sma*I site of the *E. coli* pUC18 vector. Full-length cDNA clones for all four variable genes were identified by hybridization with oligonucleotide probes and sequenced by the dideoxy method [17].

Generation of the DA4.4 and Bet 2 sFv expression plasmids

Modifications were introduced into the V_L and V_H sequences by site-directed mutagenesis in order to facilitate the insertion of the 212 linker and to allow insertion of the sFv gene in the *E. coli* pGX5410 expression vector as previously described [5] with minor modifications. In the Bet 2 V_L, a

silent codon change was made to remove a naturally occurring *Hin*dIII site at amino acid residue 38. In both DA4.4 and Bet 2 V_H clones, a *Pvu*II site was introduced at the N-terminus and two termination codons followed by a *Bam*HI site were added to the C-terminus. In the engineered Bet 2/212 sFv gene, the first two N-terminal V_L amino acid residues (N,I) have been omitted and the *Aat*II site (encoding D, V) replaces V_L residues 3 and 4 (P,L). A DNA sequence confirmation of the final constructions pGX8661 (DA4.4/212) and pGX5522 (Bet 2/212) was performed by dideoxy sequencing [17].

The pGX5410 expression vector used for sfV protein expression in *E. coli* contains the hybrid lambda phage promoter O_L/P_R and the ompA signal sequence [16]. The expression vectors pGX5522 and pGX8661 were transformed into *E. coli* host GX6712 to give the final expression strains GX8923 (Bet 2/212) and GX8662 (DA4.4/212), respectively. GX8923 and GX8662 were grown at 30 °C and the expression of the sFv proteins was induced at 42 °C. As with previously tested sFv proteins [16], the DA4.4/212 and Bet 2/212 proteins were expressed as insoluble proteins at 5–10% of total cell protein. The sFv protein preparations were analyzed for purity and integrity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) after reduction by 2-mercaptoethanol.

Renaturation and purification of anti-IgM sFv proteins

A previous protocol [5,14,16] was followed with the following modifications. *E. coli* cell paste from a 10-L fermentation was suspended in 2 L of 50 mM Tris-HCl, 1 mM EDTA, 0.3 M NaCl, 0.1 mM PMSF, pH 8.0 and was passed through a Manton–Gaulin homogenizer (APV Gaulin Corp., Wilmington, MA, USA). The cell lysate was centrifuged at 24 300 × *g* for 30 min at 6 °C. The resultant pellet was solubilized in 600 ml of 6 M guanidine hydrochloride (GuHCl) in 50 mM Tris-HCl, 10 mM $CaCl_2$, 50 mM KCl, 0.1 mM PMSF (pH 8.0) at 22 °C. The suspension was centrifuged at 24 300 × *g* for 45 min at 6 °C and the pellet was discarded. The supernatant was poured gently into 50 L refolding buffer (50 mM Tris-HCl, 10 mM $CaCl_2$, 50 mM KCl, 0.1 mM PMSF, pH 8.0 and left unstirred for 20 h at 4 °C. The refolded material was then filtered through a 0.45-μm membrane (Pellicon, Millipore Corp., Bedford, MA, USA), concentrated to 500 ml on a 10 000 molecular weight cut-off membrane and buffer exchanged into 20 mM MOPS, 0.2 mM calcium acetate, pH 6.0 for the DA4.4/212 sFv or 20 mM MES, 0.3 mM $CaCl_2$, pH 6.0 for the Bet 2/212 sFv. The resultant solutions were filtered through a 0.22-μm membrane.

For the purification of the DA4.4/212 sFv, the 0.22-μm membrane filtrate was chromatographed on a PolyCat A column (Poly LC Inc., Columbia, MD, USA) (21 mm × 250 mm) using a linear gradient of 40 mM MOPS, 1 mM calcium acetate pH 6.0 and 40 mM MOPS, 100 mM calcium acetate pH 7.0. The overall recovery was 8.5 mg of DA4.4/212 sFv protein per starting liter of culture with a purity >90%.

For the Bet 2/212 sFv, the filtrate was chromatographed on a Waters (Waters Corp., Milford, MA, USA) Accell cation-exchange (RCM) column (0.8 cm × 10 cm) which was eluted with a step gradient made up of 40 mM MES, 1 mM $CaCl_2$ pH 6.0 and 40 mM MES, 0.1 M $CaCl_2$ pH 7.0. The fractions containing material with over 90% purity were pooled, concentrated and used in subsequent assays. The observed yield was 2.6 mg of Bet 2/212 sFv protein per starting liter of culture.

Purification of anti-IgM monoclonal antibodies

The DA4.4 hybridoma cell line, obtained from the American Type Culture Collection in Rockville, MD, USA (ATCC HB 57), produces a mouse monoclonal IgG1 kappa antibody which binds to the human mu chain [12]. The Bet 2 hybridoma cell line, also obtained from ATCC (ATCC HB 88), produces a rat IgG1 kappa monoclonal antibody which recognizes a common determinant on mouse IgM [10]. Both of these cell lines were propagated in RPMI 1640 medium supplemented with heat-inactivated fetal bovine serum, L-glutamine, penicillin and streptomycin sulfate.

To produce anti-IgM monoclonal antibodies in mice, ten 4 to 6 week-old pristane primed Balb/C or nude/CRL mice were injected intraperitoneally with two million DA4.4 or Bet 2 cells, respectively. These cells had been washed free of serum proteins before injection. Ascites fluids were harvested after 60 days. Purification of the Mabs was performed on Protein G-agarose (Genex Corporation, Gaithersburg, MD, USA).

Activity determination for the anti-IgM proteins

The specificities of the sFv proteins were verified by competitive ELISAs in which the Mab was competed against its sFv counterpart for binding to the corresponding IgM immobilized on a microtiter plate. Before the competition experiment was performed, a dose response curve was constructed for Mab binding to the corresponding IgM. This identified the Mab concentration at which binding is most sensitive to the presence of competitor molecules.

IgM (10–25 ng per 100 μl per well) in 0.05 M bicarbonate buffer at pH 9.6 was added to a microtiter plate (MaxiSorp, Nunc, VWR Scientific, Boston, MA, USA) and incubated at room temperature for 1 h. The plate was then washed four times with PBS containing 0.05% Tween 20 (PBS-T). The corresponding anti-IgM Mab was serially diluted with PBS-T onto the coated wells and incubated at room temperature for 1 h. After the plate was washed, the bound Mab was detected with a secondary antibody (goat)-horseradish peroxidase (HRP) conjugate which is specific for the Fc region of the IgG (Jackson ImmunoResearch Lab., Inc., West Grove, PA, USA), and does not have affinity for the sFv protein. After 30 min at room temperature, the plate was washed with PBS-T and the HRP substrate 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS, Southern Biotechnology Associates, Inc., Birmingham, AL, USA) in citrate buffer at pH 4.0 was added to the wells for color development. The increase in absorbance at 405 nm was monitored. When a biotinylated Mab was used, the signal was obtained from a streptavidin–HRP conjugate instead of a goat IgG–HRP conjugate.

For the competition experiments, the plate was coated with IgM and washed with PBS-T as previously described. Mixtures of the Mab and varying concentrations of the sFv protein competitor were added to the coated wells and incubated at

room temperature for 1 h. After the plate was washed four times with PBS-T, either the secondary antibody–HRP or streptavidin–HRP conjugate was added to the wells and incubated for 30 min. Following buffer washes, ABTS substrate in citrate buffer pH 4.0 was added to each well for color development.

RESULTS AND DISCUSSION

Genetic construction and expression

Following cDNA cloning of the V_L and V_H genes from anti-human IgM Mab DA4.4 and anti-mouse IgM Mab Bet 2, single chain Fv protein versions of these antibodies were constructed as described in Materials and Methods. Figure 1(A) presents the sequence of the completed Bet 2/212 gene and its translation product. The 14-amino acid residue 212 linker segment in the V_L-212-V_H construction has been previously utilized [1,14] in sFv molecules and was also chosen for the constructions reported here. Figure 1(B) displays the DA4.4/212 sequence and its translation product. The DA4.4 variable genes derived from mouse, and the Bet 2 variable genes derived from rat conform to the alternating pattern of framework and complementarity-determining-regions (CDRs) predicted from the data base of variable region sequences [9]. The expression plasmid shown in Fig. 2 summarizes the engineered restriction sites and the organization of the genetic expression elements.

As with previously tested sFv proteins, the DA4.4/212 and Bet-2/212 constructions were expressed as insoluble proteins at 5–10% of total cell protein. SDS-PAGE analysis indicated that the sFv proteins exhibited the expected molecular weight of 26 000. Refolding and purification of the sFv proteins was performed as described in Materials and Methods.

Specificity determination for the DA4.4/212 sFv protein

In an experiment designed to compare the affinities of the DA4.4 Mab and sFv protein for human IgM, the DA4.4 Mab was biotinylated to act as the tracer molecule with which either the unlabeled Mab or the sFv protein would compete. Before the competition experiments were performed, the activity of the biotinylated Mab was established by a direct binding ELISA. A dose response curve was constructed (Fig. 3(A)). The concentration of the biotinylated Mab at which 50% saturation was obtained is 2.5×10^{-10} M. This is similar to that obtained when unmodified Mab was titrated against human IgM under similar conditions (Fig. 3(B)). In this case, the estimated half saturation concentration is 3.3×10^{-10} M. Therefore, biotinylation did not block the interaction between the DA4.4 Mab and human IgM.

The competition of the biotinylated Mab (2×10^{-9} M) against unlabeled Mab and the sFv protein are shown in Fig. 4(A and B respectively). Both the Mab and sFv protein competed off the labeled Mab in similar dose response curves. The specificity of the DA4.4/212 sFv protein for human IgM was further established in an experiment where an anti-fluorescein sFv protein (4-4-20/212) was used as a control and showed no competition against the DA4.4 Mab for binding to human IgM. The DA4.4/212 sFv protein, on the other hand, competes with its Mab counterpart in a dose-dependent manner.

Specificity determination of the Bet 2/212 sFv protein

The specificity of the Bet 2/212 sFv protein was verified by a competitive ELISA in which the Bet 2 Mab was competed against its sFv protein counterpart for binding to mouse IgM. When a dose response curve was constructed for binding of the Bet 2 Mab to mouse IgM, the concentration at half saturation is about 2×10^{-8} M which was the concentration then used in the competition experiment. Since the goat IgG–HRP conjugate has no affinity for the sFv protein, the signal observed is due to the binding of the Bet 2 Mab to mouse IgM. The results are summarized in Fig. 5. A Fab fragment from an unrelated rat IgG was used as a control and showed no competition against the Bet 2 Mab for mouse IgM. The Bet 2/212 sFv protein, on the other hand, competed in a dose-dependent manner. Therefore, the Bet 2/212 sFv protein exhibits a specificity for mouse IgM similar to the Mab from which it was derived.

These IgM-binding sFv proteins might now be applied to the investigation and purification of human and mouse IgM. Many antibodies of the IgM isotype are presently being excluded from research since it is difficult to recover them in a pure and active form. A slightly decreased affinity of the sFv proteins for IgM when compared to the parent Mab may be an advantage in some applications since milder conditions can be used for elution of the bound IgM. This will lessen the chance of denaturation of the IgM molecule which is relatively labile. Continued improvements in sFv fermentation yields from several milligram per liter amounts to nearly gram per liter levels may eventually allow engineered antibody fragments to be a viable commercial alternative to hybridomas.

Site-directed mutagenesis experiments can be readily performed on sFv proteins to define and refine the interaction sites. Potential uses of the DA4.4/212 and Bet 2/212 sFv proteins in research, diagnostic, therapeutic and separation applications may require or benefit from further engineered refinements in these proteins with respect to stability, affinity, and immobilization sites. The successful engineering of the DA4.4/212 and Bet 2/212 sFv proteins is a new approach to an unmet need and the long-standing goal [11] of the identification, development and production of IgM-binding proteins from bacteria.

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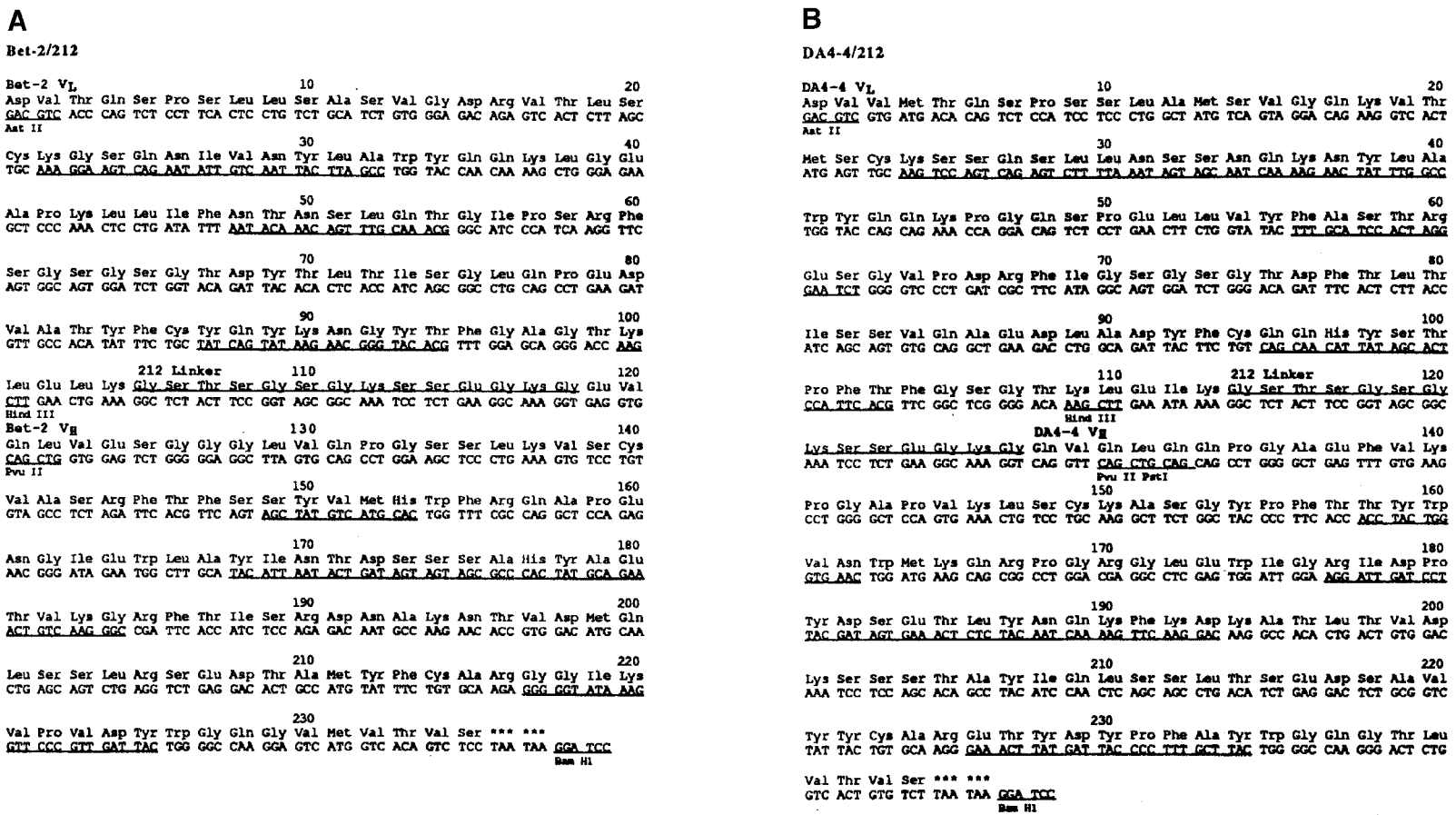


Fig. 1. The sequence of the completed sFv genes and their translation products for the (A) Bet 2/212 sFv and (B) DA4.4/212 sFv proteins. CDR sequences and the 212 linker sequence are underlined. GenBank accession numbers are L17036 and L17037 for Bet 2 and DA4.4, respectively.

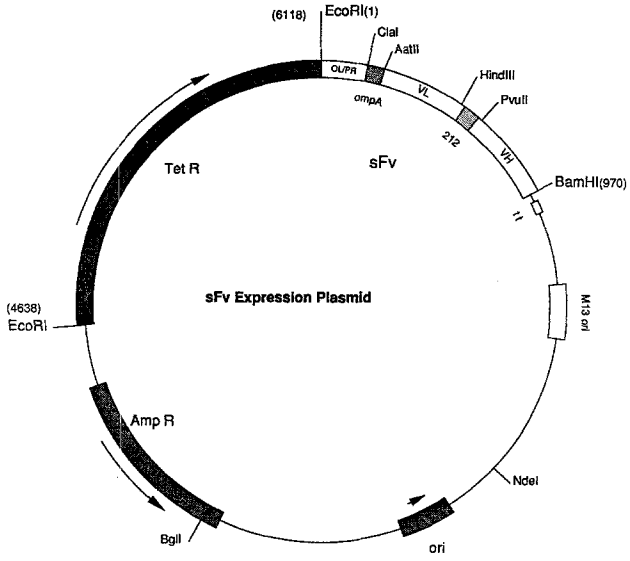
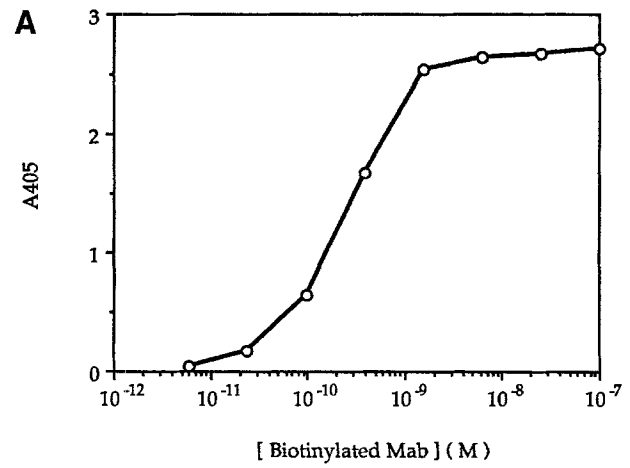


Fig. 2. A schematic diagram of the expression plasmid showing the engineered restriction sites and the organization of the genetic expression elements.



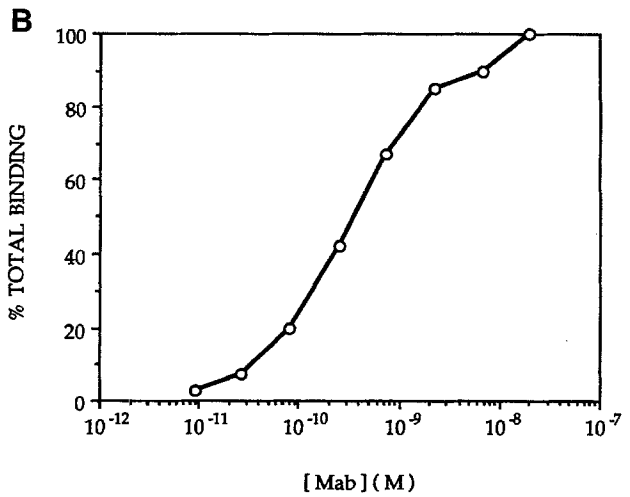
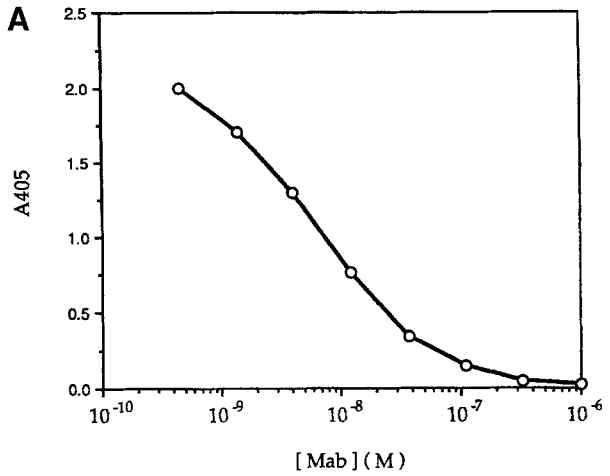


Fig. 3. Dose response curve of (A) biotinylated DA4.4 Mab and (B) native DA4.4 Mab to human IgM.



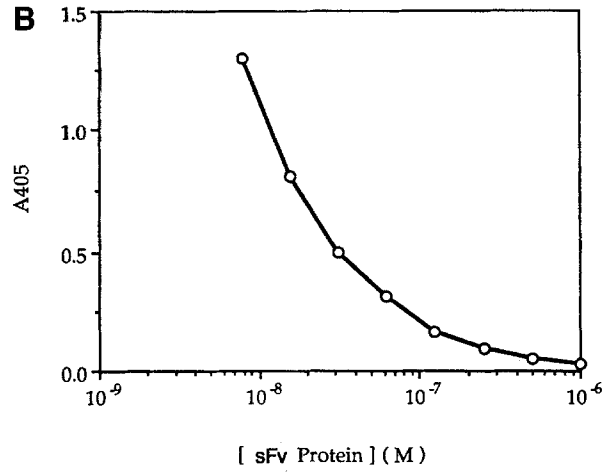


Fig. 4. ELISAs showing the competition for immobilized human IgM between biotinylated DA4.4 Mab and either (A) unlabeled DA4.4 Mab or (B) unlabeled DA4.4/212 sFv protein.

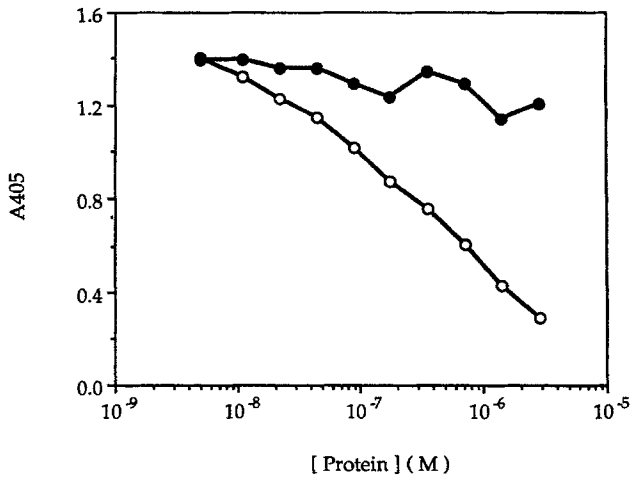


Fig. 5. An ELISA showing the competition between the Bet 2 Mab and either the Bet 2/212 sFv protein (○) or a rat control Fab (●) for immobilized mouse IgM.

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